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263/PPIR1165US

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371

U.S. APPLICATION NO.
(if known, see 37 CFR 1.5)
NEW 09/155452

International Application No.	International Filing Date	Priority Date Claimed
PCT/GB97/00875	March 27, 1997	April 1, 1996

Title of Invention
MEIOTIC RECOMBINATION IN [illegible] OF PARTIALLY HOMOLOGOUS DNA SEQUENCES

Applicant(s) For DO/EO/US
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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.

2. [] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.

3. [] This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S.C. §371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau). **ATTACHMENT A**
   b. [X] has been transmitted by the International Bureau.
   c. [] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [] A translation of the International Application into English (35 U.S.C. §371(c)(2)).

7. [] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3)).
   a. [] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [] have been transmitted by the International Bureau.
   c. [] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [] have not been made and will not be made.

8. [X] An unexecuted oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)). **ATTACHMENT B**

9. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).

Items 10. to 13. below concern other document(s) or information included:

10. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98. **ATTACHMENT C**

11. [] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

12. [X] A FIRST preliminary amendment. **ATTACHMENT D**

    [] A SECOND or SUBSEQUENT preliminary amendment.

13. [] Other items or information:

COPY, FINNEGAN [illegible] USPTO
CHARGE MISC. FEES
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THE COMMISSIONER IS AUTHORIZED TO CHARGE ANY DEFICIENCY IN THE FEES FOR THIS PAPER TO DEPOSIT ACCOUNT NO. 23-0975.

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U.S. APPLICATION NO. (if known, see 37 CFR 1.5) NEW	INTERNATIONAL APPLICATION NO. PCT/GB97/00875	ATTORNEY'S DOCKET NO. 263/PPIR1165US

14. [X] The following fees are submitted	CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):		
[X] Search Report has been prepared by the EPO or JPO..... $ 930.00		
[] Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... $1,070.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$930.00	
Surcharge of $130.00 for furnishing the oath or declaration later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

Claims	Number Filed	Number Extra	Rate		
Total Claims	-20 =		X $22.00	$	
Independent Claims	- 3 =		X $82.00	$	
Multiple dependent claim(s) (if applicable)			+ $270.00	$	
TOTAL OF ABOVE CALCULATIONS =				$930.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28)				$	
SUBTOTAL =				$930.00	
Processing fee of $130.00 for furnishing the English translation later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$930.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40 per property +				$	
TOTAL FEES ENCLOSED =				$930.00	
				Amount to be refunded	$
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a. [X] A check in the amount of $930.00 to cover the above fees is enclosed A duplicate copy of this form is enclosed.

b. [] Please charge my Deposit Account No. 23-0975 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

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NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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MEIOTIC RECOMINATION IN VIVO OF PARTIALLY HOMOLOGOUS DNA SEQUENCES

Introduction

Genetic recombination is dependent on the formation of a near perfectly paired heteroduplex joint molecule containing complementary strands from two homologous DNA duplexes. Reduced homology between substrate molecules decreases the efficiency of recombination. A striking example of this can be seen during interspecific crosses between *Escherichia coli* and *Salmonella typhymurium*. The genomes of these two bacterial species are diverged by approximately 16%. At this level of heterology the frequency of recombination during conjugational crosses is reduced by up to 5 orders of magnitude. The barrier to recombination is largely dependent on the activity of the mismatch repair system. The "disrupted species barrier" and "chromosomal instability" phenotypes, seen in bacterial mismatch repair mutants, are thought to result from a failure to prevent interactions between homeologous (closely related but non-identical) DNA sequences. This process has been termed antirecombination, although its molecular basis remains unclear. The existence of this activity has led to the proposal that the mismatch repair system is involved in controlling the fidelity of genetic exchanges. By only permitting crossovers between truly homologous sequences, such a process would suppress ectopic interactions between dispersed homologous sequences and thereby avoid potentially lethal chromosome rearrangements. Hence, the recognition of mismatches in duplex DNA may play a role in maintaining the structural integrity of chromosomes.

Many of the elements of the long-patch mismatch repair system that are believed to be involved in antirecombination have been

conserved throughout evolution. Multiple homologues to the bacterial genes *MutS* and *MutL* have been identified in organisms ranging from yeast to man. To date six *MutS* and three *MutL* homologues have been identified in S. *cerevisiae*. *PMS1* and *MSH2* are yeast homologues of the bacterial mismatch repair genes *MutL* and *MutS* respectively. A dramatic increase in the frequency of the post-meiotic segregation of genetic markers is observed in *pms1* and *msh2* mutants. This is indicative of unrepaired heteroduplex, suggesting a role for these genes in the process of gene conversion. *pms1* and *msh2* mutants also have a mutator phenotype similar to that of bacterial mismatch repair mutants. This reflects a deficiency in repair of DNA synthesis errors and spontaneous DNA lesions. Both gene products have also been shown to form part of a ternary complex that assembles *in vitro* at mismatched base-pairs in duplex DNA.

Antirecombination has profound implications for the process of meiosis. During meiotic prophase the formation of a physical connection between homologues, in the form of a crossover, allows correct orientation on the meiosis I spindle. This ensures the faithful disjunction of chromosomes to produce viable, haploid gametes. Mutations that reduce or abolish meiotic crossing over cause low spore viability, presumably due to extensive chromosomal nondisjunction. In yeast, as in bacteria, reduced chromosomal identity (~10-30% DNA sequence divergence) acts as a barrier to recombination and during meiosis, dramatically reduces exchange between homologues. The repression of recombination between homologous chromosomes during meiosis may lead to the reproductive isolation of populations in the form of sterility.

Alani *et al.*, 1994 (*Genetics* **137**: 19-39) describe interaction between mismatch repair and genetic recombination in non-homeologous situations in yeast. On the basis of an increased gene conversion frequency in msh2 mutants, the authors speculate that mismatch repair

proteins such as that coded for by msh2 might be involved in preventing homeologous recombination, but have no evidence to support this.

Prolla *et al.*, 1994 (*Molecular and Cellular Biology* **14**: 407-415). describe the identification of a new gene, MLH1, and its role in mismatch repair. The authors measured the effect of the mutants on non-homeologous simple sequence repeats and single base mismatches.

European patent specification 449 923 of Setratech is directed to a process of intergeneric recombination *in vivo* of partially homologous DNA sequences having up to 30% of base mismatches, characterized in that the sequences are placed together in cells or an

organism of which the enzymatic mismatch repair system is defective or has been inactivated transitorily, particularly by saturation, for a time to obtain recombination between the DNA sequences. Although the specification envisages the possibility of performing such recombinations in bacteria, yeasts, plant or animal cells, in fact the experimental data provided only demonstrate such recombinations in bacteria of different genera, where the recombinations are achieved by a process of mitotic recombination.

In eukaryotes, the enzymatic mismatch repair systems are more complex than in prokaryotes. Also, the enzymatic mismatch repair systems involved in meiosis are to some extent different from those involved in mitosis. It was therefore not predictable that the technique generally described in the aforesaid European patent specification could be successfully applied to eukaryotic cells undergoing meiosis.

The present invention provides process for the meiotic recombination *in vivo* of partially homologous DNA sequences having up to 30% of base mismatches, wherein eukaryotic cells containing the sequences and in which an enzymatic mismatch repair system is defective, are maintained under conditions to effect meiosis. Preferably hybrid genes and their coded proteins are formed by the process.

Preferably the process is performed for making hybrid eukaryotic species by: providing a set of first eukaryote cells containing a first DNA sequence and in which an enzymatic mismatch repair system is defective; providing a set of second eukaryotic cells containing a second DNA sequence that is partially homologous by having up to 30% base mismatches with the first DNA sequence and in which an enzymatic mismatch repair system is defective; mixing the two sets of cells to form diploids, maintaining the mixture under conditions to effect meiosis, and recovering cells of a hybrid eukaryotic species.

Although the method is applicable in principle to eukaryotes

generally, it is expected to be of particular interest in relation to plants and unicellular organisms, such as protozoa, fungi, and particularly yeasts.

The invention provides a quick and convenient way of making hybrid eukaryotic species. By suitable marking and selection, it should be possible to make hybrids having improved characteristics, e.g. the desired characteristics of both parents. For example, current brewing yeasts are difficult to work with, and the process of the present invention may result in the production of hybrid strains that are easier to work with.

To test this proposal a hybrid of the bakers yeast *S. cerevisiae* and its sibling species *Saccharomyces paradoxus* has been utilized. *S. paradoxus* (also described as *Saccharomyces douglasii*) is the closest relative of S. *cerevisiae* isolated to date. Electrophoretic karyotyping and hybridization analysis reveal that the genomes of the two species are very similar in terms of chromosome number, size and the location of genes. The weak hybridization of many cloned *S. cerevisiae* genes with *S. paradoxus* chromosomes demonstrates that DNA divergence exists between the two species. From the limited DNA sequence data available, divergence has been estimated to be ~11% and ~20% in coding and non-coding regions respectively. A hybrid of *S. cerevisiae* and *S. paradoxus* therefore comprises genome-wide homology but appears to lack major structural differences in karyotype.

The following experimental report examines meiotic recombination and chromosome disjunction in this hybrid and the effects of the mismatch repair genes *PMS1* and *MSH2* on these processes.

Results

Experimental Rationale

The model depicted in Figure 1 predicts that meiosis in nuclei containing divergent parental genomes will be associated with both low frequencies of reciprocal exchange and high frequencies of chromosome

nondisjunction. This will lead to low viability of the meiotic products (reduced fertility). In the absence of mismatch recognition, crossovers will be permitted between homologous chromosomes, disjunction will be improved and a greater number of viable, euploid gametes will be produced.

The S. cerevisiae/S. paradoxus Hybrid

A wild-type, homothallic isolate of *S. paradoxus*, NI7, has been engineered into a genetically tractable organism (see Materials and Methods). Hybrids of N17 and the *S. cerevisiae* strain Y55 produce only 1% viable spores (Table I and II, strain NHD47). Many of these are have abnormal cell and colony morphologies and are slow growing, often producing only a microcolony. This sterility has been noted in similar hybrids and forms the basis of the biological species definition of yeast taxonomy.

The low spore viability of the hybrid is expected to be associated with high rates of chromosome nondisjunction. To test this prediction meiotic chromosome nondisjunction was monitored by physical analysis of the karyotypes of random spores. Separation of the yeast chromosomes by Clamped Homogenous Electric Field (CHEF) gel electrophoresis allows the assignment of disomy for the ten smallest *Saccharomyces* chromosomes. In the hybrid the frequency of disomy is high for all the chromosomes analyzed (Table III) with the exception of chromosome VI, which is always monosomic (see Discussion). Nondisjunction rates are up to 500-fold higher than that of a *S. cerevisiae* intraspecic diploid. The meiosis I nondisjunction rates of chromosomes IV and XI have been measured by a genetic analysis of random spores in a *S. cerevisiae* Y55 strain at 1.4 x10-4 and 5.0 x 10-4 per meiosis, respectively. Chromosome II exhibited the highest rate of nondisjunction in the wild-type hybrid at 2.7 x 10^{-1} per meiosis. The distribution of disomes

closely fits that expected from an average nondisjunction rate of 12.2%, for the nine chromosomes examined (see Data Analysis). If this calculation is extended to all sixteen *Saccharomyces* chromosomes we expect 12.5% of spores to have no disomes, 27.7% to have one, 22.9% two and 36.9% to have three or more disomes.

The primary cause of chromosome nondisjunction is proposed to be low frequencies of genetic exchange. To ascertain recombination frequencies random spores were monitored for recombination in four genetic intervals: *HIS4-LEU2* and *LEU2-MAT* on chromosome III, *TRPI-ADE8* on IV and *CYH2-MET13* on VII (Table IV). The frequency of recombination is 11.5 to 79-fold reduced relative to the intraspecific control *S. cerevisiae* diploids (NHD50, 53 and 94). The *TRPI to ADE8* interval demonstrates a profound reduction in map distance. This large genetic interval is 270 cM in *S. cerevisiae*. In the hybrid the markers are tightly linked, with a map distance of approximately 2 cM.

Mismatch-Repair Deficient Hybrids

To examine the effect of the mismatch repair system on meiosis in *S. cerevisiae /S. paradoxus* hybrids, we disrupted the *PMS1* and *MSH2* genes in haploids of both species to produce the hybrid diploids NHD45 and NHD94.

Spore viability is significantly improved in the *pms1* and *msh2* hybrids by 6.1 and 8.7-fold respectively (Table II). Moreover, the accumulation of haplo-lethal mutations due to the mutator phenotypes of *pms1* or *msh2* produce ~ 21% spore death in intraspecific diploids (Table II, strains Y55-518, Y55-512, NHPD1 and NHPD2). Therefore, correcting for the death induced by mutation, the viability of the hybrids can be estimated to be 7.4 and 11.5-fold greater than that of the wild-type hybrid. The difference between the viability of the *pms1* and *msh2* hybrids is also significant. Additionally, it was noted that viable spores from *msh2* hybrids

are less abnormal in colony morphology and are faster growing, forming fewer microcolonies. This may be a direct phenotypic manifestation of lower levels of aneuploidy.

Reduced Aneuploidy in Mismatch Repair Mutants

The improvement in spore viability in the mismatch repair deficient hybrids is concomitant with significant reductions in disomy. In both the mutant hybrids there is an improvement in the disjunction of all the chromosomes analyzed (Table III). In the *pms1* hybrid the total frequency of disomes is reduced 1.8-fold over the wild type hybrid. The improvement in disjunction is even greater in the *msh2* hybrid, with a further 1.8 fold reduction in total disomes. This indicates a significant disparity between the *pms1* and *msh2* mutants with respect to chromosome disjunction. In addition, the distribution of disomes between the three hybrid diploids is significantly different. In the wild-type hybrid only 32% of spores are not disomic for any of the nine chromosomes analyzed and nearly 12% contain three or more disomes. By comparison, 70% of the spores from the *msh2* hybrid have zero disomes and no spores contain more than two disomes.

Recombination is Increased in pms1 and msh2 Hybrids

Genetic analysis of random spores from the *pms1* mutant hybrid reveals a 2.3 to 10-fold increase in recombinants for the four intervals monitored. As might be expected from the disjunction data, the effect of the *msh2* disruption is greater, producing a 6.0 to 16.5-fold increase in recombinant frequency. Again this reflects a significant difference between the two mutant hybrids. No change in recombinant frequency is observed in the *pms1* and *msh2*, intraspecifc *S. cerevisiae* diploids (NHD53 and NHD95), demonstrating that the observed effects are specific to the hybrids. The improved spore viability of the *msh2* hybrid permitted limited "tetrad analysis" to be performed. Out of 53 tetrads with one or more viable

spores, 3.9% have a recombination event in the interval *HIS4-LEU2*, 19.6% between *LEU2-MAT*, 41.2% in *TRPI-ADE8* and 3.8% between *CYH2-MET13*. These frequencies are not statistically different to those obtained from the analysis of random spores. In addition, *bona fide* reciprocal events in three of the four intervals analyzed *(LEU2-MAT, TRPI - ADE8* and *CYH2-MET13)* were represented in the tetrads with two or more viable spores.

Meiotic crossing over is reduced between homeologous chromosomes.

Meiotic recombination in the partial hybrid can be monitored in four genetic intervals, covering approximately 250 kb of the 320-kb chromosome III. The frequency of exchange between the divergent chromosomes was determined by tetrad analysis. Recombination data were calculated from tetrads with four viable spores and from asci which yielded only three viable spores. For this latter class of tetrad, it is possible to predict the genotype of the dead spore from the segregation pattern of genetic markers observed in the remaining viable spores. Data from these two classes of tetrad are presented in Table V, and map distances are shown in Table VI. Recombination data for homologous mismatch repair-deficient strains were not determined, because previous control experiments have demonstrated that *pms1* and *msh2* mutants do not affect the rates of intergenic, meiotic recombination in a perfectly homologous diploid. Crossing over in the partial hybrid is suppressed in each of the four genetic intervals monitored. The *HML*-to-*HIS4* map distance is contracted 60-fold when compared with that of the control Y55 homozygous diploids. A 47-fold reduction in exchange is observed in the *MAT-THR4* interval. Only one event is observed in the *HIS4-LEU2* region in the 440 tetrads analysed. The smallest reduction is in the *LEU2-MAT* interval, which exhibits a nine-fold reduction in exchange. The average

reduction over the entire interval from *HML* to *THR4 is* 25-fold. The overall reduction is even more extreme, 40-fold, if we consider only the four-viable-spore tetrad class (Table V).

Mismatch repair mutant partial hybrids are predicted to show elevated frequencies of recombination. A partial-hybrid diploid, homozygous for a deletion of the *pms1* gene, was constructed and tetrads were analysed (Tables V and VI). Reciprocal exchange is increased in three of the four intervals monitored; over the combined *HML*-to-*THR4* region, there is a 2.5-fold increase over that in the wild-type partial hybrid. However, no stimulation of recombination is observed in the *MAT-THR4* region. Overall, the *HML-to-THR4* map distance remains more than 10-fold reduced relative to the homologous controls. An *msh2* partial hybrid was also constructed (Tables V and VI). Recombination in this strain is affected to a greater extent than in the *pmsl* diploid. The map distance is expanded 5.5-fold in the *HML-THR4* interval relative to that in the wild-type partial hybrid. This represents a 4.5-fold reduction in recombination relative to the homologous controls. The frequency of exchange observed in the *msh2* mutant is significantly greater than in the *pmsl* partial hybrid. Genetic exchange in a *pms1 msh2* double mutant (Tables V and VI) increases significantly compared with that in the *pms1* and *msh2* partial hybrids ($P < 0.001$ and $P < 0.01$, respectively) over the whole *HML-THR4* interval. The map distance increases by 7-fold in the double mutant relative to the wild-type partial hybrid and is only 3.5-fold reduced from the homologous control. The increase in map distance in the double mutant is the sum of the increases in the single mutants. The map distance of *HML-THR4 is* 34.7 centimorgans (cM) in the double mutant compared with 11.8 and 26.6 cM in the *pms1* and *msh2* single mutants respectively. These properties of the double mutant, compared to the single mutants, are completely unexpected.

Discussion

The Meiotic Behavior of an Interspecific Yeast Hybrid Satisfies the Predictions of the Antirecombination Model

The frequency of meiotic recombinants are reduced to between 1.3% and 8.7% of intraspecies frequencies over four genetic intervals that vary from 11 to 270 cM. The reduction is greatest over the largest region, *TRP1* to *ADE8*. The map distance is reduced ~136-fold, predicting that a crossover in this interval will occur in less than 4% of meioses. The low recombination rates confer a several hundred-fold increase in the frequency of nondisjunction. However, we would have expected such low frequencies of recombination to be associated with an greater nondisjunction rate. For example, a 20-fold reduction in map distance for a 200 cM chromosome will result in a frequency of homologue pairs without reciprocal exchange (E_o) of 82% (see Data Analysis). If E_o homologues then segregate randomly at meiosis I, 41 % will nondisjoin. Such a high frequency of disomy is not seen for any of the chromosomes analyzed, although greater than 20-fold reductions in recombination do occur. There are a number of explanations which could account for this observation. Firstly, the viable random spores may be underrepresented for aneuploidy because particular combinations of disomes are either lethal or produce slow growing colonies. In this study chromosome VI was never found to be disomic in over three hundred CHEF karyotypes. This could be because VI disomy is lethal. While this is not the case for intraspecific *S. cerevisiae* cells, in which VI disomy is tolerated, it is possible the *S. cerevisiae* and *S. paradoxus* chromosomes could be incompatible. Alternatively, it is possible that chromosome VI always disjoins correctly. The existence of a distributive pairing mechanism may also account for lower than expected levels of aneuploidy. Distributive pairing improves the segregation of E_o and heterologous chromosomes. For example, a pair of

heterologous yeast artificial chromosomes (YACs) do not recombine at detectable frequencies but only missegregate in 25% of meioses, not the expected 50%. Finally, the divergence between the two yeast species may be mosaic in nature. The degree of identity along the chromosomes and between different chromosomes may vary widely. Regions of high homology, that frequently recombine to ensure disjunction, could be present.

The Mismatch Repair Proteins PMS1 and MSH2 Reduce Meiotic Homologous Recombination

The *pms1* mutation restores meiotic recombination in the hybrid to between 9% and 27% of intraspecies frequencies. In *msh2* hybrids the frequency of recombinants is 20% to 69% of the homologous controls. The increase in *TRP1-ADE8* map distance in the *msh2* hybrid predicts a crossover will occur in nearly 50% of meioses. However, this is still a low frequency when compared to the intraspecies *S. cerevisiae* interval which has approximately 5 crossovers per meiosis. Recombinants are observed in spores from tetrad dissection at frequencies equivalent to those from random spore analysis. It is important to note that both products of reciprocal exchange events are recovered in the tetrads with two or more viable spores from this analysis. This indicates that random spore recombinants represent true crossover products. The *pms1* and *msh2* Y55 intraspecific, control diploids have no increase in the frequency of meiotic recombination. These controls rule out the possibility that the increase in recombinants in the mutant hybrids is due to a general hyperrecombination phenotype or to marker reversion. We conclude that the mismatch repair system actively inhibits meiotic exchange between highly divergent chromosomes.

The observation that recombination is never fully restored in mismatch repair deficient hybrids could be due to several factors. Other

mismatch repair proteins, that inhibit homologous recombination, may still be active in these mutants. Also, the degree of initiation of meiotic recombination may be reduced between homologous chromosomes. This "trans effect" of heterozygosity has been observed at two loci in *S. cerevisiae*. Also some regions of the chromosomes may be so diverged that homology is no longer recognized at the strand exchange stage of recombination (see also below).

To determine if *PMS1* and *MSH2* are operating in the same or different pathways during homeologous exchanges, a *pms1 msh2* double-mutant strain was constructed. Because Pms1p and Msh2p are proposed to act in concert, we would have predicted that a double mutant would be no more severe than either mutant alone. Analysis of post-meiotic segregation frequencies and rates of mitotic mutation support this proposal. However, with respect to meiotic homeologous recombination, the phenotype of the double mutant is more severe. The total increase in homeologous recombination in the double mutant significantly exceeds the rates of exchange observed in either the *pms1* or *msh2* strain ($P < 0.001$ and $P < 0.01$, respectively). In fact, the rates of exchange in the double mutant are additive

Crossovers Ensure Disjunction

The correlation between greater crossing over and decreased aneuploidy indicates that many of the crossovers restored in the mutant hybrids can ensure disjunction, that is, can form functional chiasmata (a cytological manifestation of crossing over). This relationship between crossover frequency and chromosome disjunction is not linear. From a comparison with *S. cerevisiae* recombination mutants we propose that the deficiency of recombination is the major reason for nondisjunction in the hybrid. For example, the *med1* mutation, an allele of the *DMC1* gene, has a 2-fold decrease in meiotic crossovers, 4.3% chromosome III and 6.6%

chromosome VIII disomes, and 20% spore viability. This is similar to the *msh2* hybrid which has approximately a 3-fold decrease in exchange, 6% chromosome III and 3% chromosome VIII disomes, and 10% spore viability.

It must also be considered that the reduced fidelity of recombination in mismatch repair deficient hybrids may increase the frequency of crossovers between related, ectopic loci. Ectopic crossovers are known to interfere with homologue disjunction and are likely to produce lethal, unbalanced translocations. One such translocation, giving rise to a unique sized chromosomal species has been observed in a segregant from the *msh2* hybrid (not shown).

Spore Viability is Improved in pms1 and msh2 Hybrids

The increased spore viability appears to be a direct consequence of improved chromosome disjunction which in turn is the result of increased frequencies of meiotic recombination. The spore viability of the hybrids are lower than expected from the patterns of disomy observed. The average frequency of disomes in random spores from the *msh2* hybrid is 3.7% per chromosome. The observed frequency of 70% spores with no disomes, for the nine chromosomes examined, closely fits the expected frequency . If this rate of disomy is assumed for all sixteen *Saccharomyces* chromosomes, the expected number of spores with no disomes is 55%. Therefore the minimum expected spore viability for the msh2 hybrid is 55%. The fact that spore viability is not restored to this level indicates that other factors probably contribute to the meiotic sterility of the *S. cerevisiae/S. paradoxus* hybrid. The observation that some *S. paradoxus* chromosomes are haplo-insufficient in an otherwise *S. cerevisiae* genetic background, suggests that chromosomal rearrangements or incompatibilities, that could contribute significantly to spore inviability, may be present between the two species.

In summary, an active mismatch repair system reduces meiotic exchange between divergent chromosomes, increases their rate of nondisjunction and reduces spore viability.

Processing of Mismatched Recombination Intermediates

How the mismatch repair system processes mismatched recombination intermediates at the molecular level is not clear. Several models have been proposed. The "killer mechanism" causes the destruction of intermediates which could potentially lead to chromosomal loss. Mismatch repair-induced recombination may lead to chromosomal rearrangement or loss. Lastly, antirecombination and the similar "heteroduplex rejection" models propose that intermediates are aborted via disassembly, or resolution without exchange. From the data presented here none of these possibilities can be excluded. However, the low frequencies of meiotic exchange and high levels of aneuploidy are most consistent with an antirecombination mechanism.

Several observations, from a variety of experimental approaches, suggest that recombination intermediates are disrupted at an early stage, prior to the formation of a stable heteroduplex junction. Firstly, individual components of the bacterial mismatch repair system can block *in vitro* homologous strand exchange catalyzed by the *E. coli* RecA protein. Also recombination intermediates that have been detected during meiotic prophase I, in *S. cerevisiae*, were not observed to form between homologous chromosomes. Finally, F1 hybrids between species of *Allium* that lack gross chromosomal rearrangements have a substantial reduction in the frequency of chiasma and an increased number of univalents at pachytene.

Mutations in the mismatch repair genes *MSH2* and *MSH3* have been shown to increase the frequency of mitotic homologous recombination, in *S. cerevisiae*, between substrates with 73% identity.

Mutation of *PMS1* had no significant effect on recombination, an observation made by other workers utilizing mitotic recombination assays with similarly diverged substrate DNA (~80% identity). However in a recent mitotic study, a 10-fold effect of *pms1* was observed with 92% identical substrates. In the data presented here, *pms1* produces up to a 10-fold enrichment in meiotic recombinants. The effect of *msh2 is* significantly greater, not only in terms of recombination, but also for disomy and spore viability. This observation suggests that the method of processing recombination intermediates depends upon the degree of divergence between the participating molecules. At relatively high levels of divergence (10-30%) *MSH2* appears to have a greater role than *PMS1* in preventing homologous recombination.

From the known biochemical properties of the *E. coli* MutS and *S cerevisiae* MSH2 proteins it is assumed that DNA divergence will be recognized when mismatches form in heteroduplex DNA. A number of features of meiotic homologous recombination follow from this assumption. The fact that reasonable frequencies of recombination are observed in *pms1* and *msh2* hybrids suggests that the induction of meiotic recombination is still high. Also, consistent with the *in vitro* properties of *E. coli* RecA protein, high densities of mismatches are not normally inhibitory to strand exchange *per se* in yeast. However, very high divergence (greater than 30% mismatches) may act as a structural barrier to strand exchange.

Materials and Methods

Strains

All *S. cerevisiae* and *S. paradoxus* strains used in this study are isogenic derivatives of Y55 and N17 respectively. Genotypes are described in Table I. The *ho-ochre* mutation was isolated by UV-mutagenesis but was found to have a slightly leaky phenotype.

Subsequently, heterothallic strains were obtained by creating a 100 bp PstI deletion of the coding sequence of the *HO* gene. The Δ *pms1* mutation is a 2.6 kb deletion of the *PMS1* coding sequence. Both ho Δ Pst and Δ *pms1* were cloned into a *URA3* selectable, integrative vector and introduced via two-step gene replacement. The *pms1* Δ ::*URA3* mutation is a *URA3* replacement of 2.6 kb of the *PMS1* coding sequence. *msh2* :: *LEU2 is* an insertion of *LEU2* at a *Sna BI* site of the wild-type gene in plasmid pII-2. Both were introduced by one-step gene transplacement. *his4-RI, trpl-bsu36* and *ura3-nco* are restriction site fill-in mutations. *leu2* Δ is a deletion of most of the *LEU2* coding region. All were introduced by two-step gene replacement. Other auxotrophic markers were spontaneous or UV-induced. All transformations were verified by Southern blot analysis using the digoxigenin, nonradioactive system as recommended by the manufacture (Boehringer Mannheim).

A *pms1 msh2* double mutant was created by two-step gene replacement with the BstXI fragment of pWK4Δpms1 followed by one-step gene transplacement of the *SpeI* fragment of pII-2-7.

The resulting strain had this genotype.

MATa HML::ADE1 his4-r leu2-r thr4-a^{b} *KAR1*

MATα HML HIS4 LEU2 THR4^{c} *kar1-Δ13*

ade1-1 can1-1 ura3-n pms1Δ msh2::URA3 CYH2 lys2-d

ade1-1 CAN1 ura3-n pms1Δ msh2::URA3 cyh2-1 LYS2

Genetic Procedures

Yeast manipulations and media were as described in the literature. Strains were grown on YPD and synthetic complete media lacking one or more amino acids, at 30°C. Sporulation was performed at room temperature on KAc plates: 2% potassium acetate, 0.22% yeast extract, 0.05% glucose, 2.5% agar 0.09% complete amino-acid mixture .

Dissected tetrads were grown for 3-7 days at 30°C. Germination was scored microscopically after 3 days. Only spores that formed microcolonies were scored as being viable. Random spores were prepared and grown on synthetic complete media lacking arginine, containing cycloheximide (10 mg/L) and canavanine (40 mg/L) for 3-6 days at 30°C. One and two-step gene replacement was performed. Yeast transformation was carried out using a modification of the lithium acetate method.

Chromosome transfer by karl

Strains that were partially hybrid for chromosome III were created by *karl*-mediated single-chromosome transfer from N17 into Y55-2395 by a modification of previously described methods. Chromosome transfer events were selected on synthetic medium lacking leucine and supplemented with cycloheximide (10 mg/litre). Strains disomic for chromosome III, which arise from the chromosome transfer event, were confirmed physically by the appearance of a band of double intensity by CHEF gel analysis and genetically by a nonmating sporulation-deficient phenotype.

Selection for loss of the resident Y55 chromosome III

S. cerevisiae strains monosomic for the N17 chromosome III were constructed by transplacing the *EcoRI* fragment of pGEM7.10ΔCX*URA*3 (a *URA*3 disruption of the *MSH*3 open reading frame) into the disomic strains obtained from chromosome transfer. Transplacement occurs preferentially into the Y55 copy of the *MSH*3 gene because of reduced homology with the *S. paradoxus* chromosome. Subsequently, 5-fluoroorotic acid selection for *ura*3⁻ strains was used to obtain haploids which had lost the resident Y55 chromosome. The

resulting monosomic strains are α maters with the *S. paradoxus* III genotype.

Karyotyping of Segregants

Random spore segregants were karyotyped. Disomy was assigned via band intensity or the presence of two bands for co-migrating and polymorphic chromosomes respectively.

Data Analysis

Data sets were analyzed using the standard normal, nonparametric sign and G-tests. The G-test is an equivalent to the X^2 contingency test. Values of $P<0.05$ were considered significant. Expected distributions of disomes were calculated using the average disomy frequencies in a binomial expansion involving 9 or 16 chromosomes. E_o values were calculated assuming recombination rates in random spores are equivalent to map distance and a poisson distribution of the number of crossovers per chromosome.

Figure Legends

Figure 1. A model of the biological consequences of antirecombination during meiosis

A. Homologous chromosomes recombine and undergo crossing over. The homologues become physically connected by a chiasmata and consequently orientate correctly on the meiosis I spindle. Correct disjunction in the first division is followed by an equational division to produce four euploid spores. Spores b. and c. contain recombinant chromosomes.

B. The mismatch repair proteins will prevent a crossover between homologous chromosomes. Apposition of the centromeres is not ensured and the resultant univalents segregate randomly with respect to each other at meiosis I. If both univalents attach to the same spindle nondisjunction will result. After meiosis n two disomic and two nullosomic spores will be produced. None of the chromosomes will be recombinant. The nullosomic cells lack essential genetic information and will be dead. The disomic cells contain unbalanced genomes and may have reduced fitness.

Table I Strains used in this study

Strain	Genotype	Reference
Y55	*S.c. HO* wild type	(McCusker, 1988)
N17	*S.p. HO* wild type	(Naumov, 1990)
Y55-518	*S.c. hoΔPst MATα arg4-1 HIS6 leu2-1 trp5-1 ura3-nco Δpms1* *S.c. hoΔPst MATa arg4-1 his6-1 leu2-1 TRP5 ura3-1 Δpms1*	This study
Y55-512	*S.c. ho-ochre MATα arg4-1 HIS6 leu2-1 trp5-1 ura3-1 msh2::LEU2* *S.c. ho-ochre MATα arg4-1 his6-1 leu2-1 TRP5 ura3-1 msh2::LEU2*	This study
NHPD1	*S.p. hoΔPst MATa CAN1 cyh2-1 lys2-1 LYS5 ura3-1 pms1Δ::URA3* *S.p. hoΔPst MATα can1-1 CYH2 LYS2 lys5-1 ura3-1 pms1Δ::URA3*	This study
NHPD2	*S.p. hoΔPst MATa CAN1 cyh2-1 lys2-1 LYS5 ura3-1 msh2:URA3* *S.p. hoΔPst MATα can1-1 CYH2 LYS2 lys5-1 ura3-1 msh2:URA3*	This study
NHD50	*S.c. hoΔPst MATa ade8-1 can1 CYH2 his4-R1 leu2Δ met13-4 trp1-bsu36 ura3-nco* *S.c. hoΔPst MATα ADE8 CAN1 cyh2-1 HIS4 LEU2 MET13 TRP1 ura3-nco*	This study

NHD53	*S.c. hoΔPst MATa ade8-1 can1 CYH2 his4-RI leu2Δ met13-4 trp1-bsu36 ura3-nco pms1Δ::URA3* *S.c. hoΔPst MATα ADE8 CAN1 cyh2-1 HIS4 LEU2 MET13 TRP1 ura3-1 pms1Δ::URA3*	This study
NHD95	*S.c. hoΔPst MATa ade8-1 can1 CYH2 his4-RI leu2Δ met13-4 trp1-bsu36 ura3-nco msh2:URA3* *S.c. hoΔPst MATα ADE8 CAN1 cyh2-1 HIS4 LEU2 MET13 TRP1 ura3-1 msh2:URA3*	This study
NHD47	*S.c. hoΔPst MATa ade8-1 can1 CYH2 his4-RI leu2Δ met13-4 trp1-bsu36 ura3-nco* *S.p. hoΔPst MATα ADE8 CAN1 cyh2-1 HIS4 LEU2 MET13 TRP1 ura3-1*	This study
NHD45	*S.c. hoΔPst MATa ade8-1 can1 CYH2 his4-RI leu2Δ met13-4 trp1-bsu36 ura3-nco pms1Δ::URA3* *S.p. hoΔPst MATα ADE8 CAN1 cyh2-1 HIS4 LEU2 MET13 TRP1 ura3-1 pms1Δ::URA3*	This study
NHD94	*S.c. hoΔPst MATa ade8-1 can1 CYH2 his4-RI leu2Δ met13-4 trp1-bsu36 ura3-nco msh2:URA3* *S.p. hoΔPst MATα ADE8 CAN1 cyh2-1 HIS4 LEU2 MET13 TRP1 ura3-1 msh2:URA3*	This study

Abbreviations: *S. c.*, *Saccharomyces cerevisiae*; *S. p.*, *Saccharomyces paradoxus*. All *S. c.* and *S. p.* strains are isogenic to the wild-type isolates Y55 and N17 resectively. Strains were constructed as described in Methods and Materials.

Table II Spore viabilities of intraspecific and hybrid yeast diploids

Strain	Genotype	Percentage spore Viability
Y55	*S. c. wt*	97.8 (841/860)
N17	*S. p. wt*	97.0 (194/200)
Y55-518	*S. c. pms1*	80.5 (679/844)
NHPD1	*S. p. pms1*	71.3 (117/164)
Y55-512	*S. c. msh2*	84.0 (776/924)
NHPD2	*S. p. msh2*	80.42 (193/240)
NHD47	Hybrid *wt*	1.2 (10/852)
NHD45	Hybrid *pms1*	7.2 (63/880)
NHD94	Hybrid *msh2*	10.2 (147/1440)

Diploids were sporulated and tetrad ascospores dissected. To reduce the spore death caused by the mutator phenotypes of *pms1* and *msh2*, vegetative growth as a diploid was minimised. Haploid strains were mated for only 6 hrs at 30°C, and the diploids were not selected prior to sporulation. All strains were treated in this way. The spore viability of all three hybrids is significantly different from all intraspecific diploids as determined by standard normal test ($P<<0.001$). The *pms1* and *msh2* hybrid viabilities are different from the wild-type hybrid ($P<0.01$ and $P<<0.001$ respectively), and the *msh2* hybrid is different from the *pms1* hybrid ($P<0.01$).

Table III Frequency of disomes in hybrid segregants

	Percentage Of Spores With Disome									
Strain	I	VI	III	IX	VIII	XI	X	XIV	II	Total
NHD47 (*wt*)	18.4 (19/103)	0.0	9.7 (10/103)	12.6 (13/103)	21.4 (22/103)	5.8 (6/103)	13.6 (14/103)	1.0 (1/103)	27.2 (28/103)	12.2 (113/927)
NHD45 (*pms1*)	13.3 (14/105)	0.0	7.6 (8/103)	5.7 (6/105)	7.6 (8/105)	1.9 (2/105)	8.6 (9/105)	0.0	14.3 (15/103)	6.6 (62/945)
NHD94 (*msh2*)	4.0 (4/100)	0.0	6.0 (6/100)	4.0 (4/100)	3.0 (3/100)	5.0 (5/100)	7.0 (5/100)	0.0	4.0 (4/100)	3.7 (33/900)

Random segregants were karyotyped by CHEF gel electrophoresis. The total numbers of disomes are significantly different between all three data sets as defined by a standard normal test ($P < 0.01$ to $P << 0.001$) and the individual data sets are different by non-parametric sign test ($P<0.05$ to $P<0.01$). The data sets for chromsomes VIII and II are different between NHD47 and NHD45 ($P<0.01$ and $P<0.05$ respectively). Chromsome I, IX, VII, X, and II data sets are different between NHD47 and NHD94 ($P<0.05$ to $P<<0.001$). The frequency of disomes for chromsomes I and II are significantly different between NHD45 and NHD94 ($P<0.01$).

Table IV Meiotic recombination

Strain	Percentage Recombinants			
	HIS4-LEU2	*LEU2-MAT*	*TRP1-ADE8*	*CYH2-MET13*
NHD50 (*S.c. wt*)	18.33 (66/360)	21.67 (78/360)	46.39 (167/400)	11.94 (43/360)
NHD53 (*S.c. pms1*)	17.22 (62/360)	24.72 (89/360)	49.44 (178/360)	11.94 (43/360)
NHD95 (*S.c. msh2*)	23.8 (86/360)	22.78 (82/360)	47.5 (171/360)	9.17 (33/360)
NHD47 (Hybrid *wt*)	0.25 (1/400)	2 (8/400)	2 (8/400)	0.25 (1/400)
NHD45 (Hybrid *pms1*)	1.75 (7/400)	4.5 (18/400)	13 (52/400)	2.5 (10/400)

NHD94 (Hybrid *msh2*)	4 (16/400)	12 (48/400)	33 (132/400)	3.5 (14/400)

Random spores were analysed for recombination in the four intervals shown. Map distance is equivalent to the frequency of recombinants. None of the intervals in the control diploids, NHD50, 53 and 95 are statistically different by standard normal test. Therefore a pool of these data sets was used for comparison to data for the hybrid diploids. All intervals in the three hybrid diploids are significantly different to intraspecific controls ($P<<0.001$). The recombinant frequency in all four intervals in the *pms1* hybrid is statistically different to the wild-type hybrid ($P<0.05$ to $P<<0.001$). Likewise all intervals are different between *msh2* and wild-type hybrids ($P<0.001$ to $P<<0.001$). Additionally, the *LEU2-MAT* and *TRP1-ADE8* data sets are different between *pms1* and *msh2* hybrids ($P<<0.001$) and the *HIS4-LEU2* data is suggestive of a difference ($P=0.056$). The total number of recombinants is also significantly different between the three hybrids ($P<<0.001$).

Table V. Total meiotic recombination in tetrads with three and four viable spores.

Strain	% Recombination in tetrad class[a] Total recombination %	
	4 spores	3 spores
Wild-type homozygote	200[b] (665/333)	185[b] (24/13)
Wild-type partial hybrid	5.04 (18/357)	28.9 (24/83)
pms1 partial hybrid	22.1 (31/140)	26.3 (20/76)
msh2 partial hybrid	47.4 (65/137)	66.1 (39/59)
pms1 msh2 partial hybrid	63.2 (72/114)	78.0 (64/82)

[a] Recombination data are pooled from two independent diploids for each cross. Numbers in parentheses represent total numbers of reciprocal exchange events including twice the number of nonparental ditypes (nonparental diploids were observed only in the wild-type homologous diploids.

[b] Each tetrad in the wild-type homologous control had more than one crossover across the whole interval monitored, hence the >100% total recombination observed.

Table VI. Genetic Map Distances

Strain	Map distance (cM) of genetic interval[a] Total *HML-THR4*	Fold Reduction
Wild-type homozygous control	120	1.0
Wild-type partial hybrid	4.8	25.0
pms1 partial hybrid	11.8[b]	10.2
msh2 partial hybrid	26.6[b]	4.51
pms1 msh2 partial hybrid	34.7[b]	3.46

[a] Map distance in centimorgans (cM) is calculated as described in Materials and Methods.

[b] Values significantly deviating from those for the wild-type partial hybrid ($P<0.001$).

CLAIMS

1. Process for the meiotic recombination *in vivo* of partially homologous DNA sequences having up to 30% of base mismatches, wherein eukaryotic cells containing the sequences and in which an enzymatic mismatch repair system is defective, are maintained under conditions to effect meiosis.

2. Process according to claim 1 wherein hybrid genes and their coded proteins are formed.

3. Process according to claim 1 for making hybrid eukaryotic species, comprising providing a set of first eukaryotic cells containing a first DNA sequence and in which an enzymatic mismatch repair system is defective; providing a set of second eukaryotic cells containing a second DNA sequence that is partly homologous by having up to 30% base mismatches with the first DNA sequence and in which an enzymatic mismatch repair system is defective; mixing the two sets of cells, to form diploids maintaining the mixture under conditions to effect meiosis, and recovering cells of a hybrid eukaryotic species.

4. Process according to claim 1, wherein the eukaryotic cells are of unicellular organisms.

5. Process according to claim 4, wherein the unicellular organisms are yeasts.

6. Process according to claim 1, wherein the enzymatic mismatch repair systems of the eukaryotic cells are defective by virtue of at least one *mutS* protein and/or at least one *mutL* protein being defective or missing.

7. Process according to claim 6, wherein the eukaryotic cells containing the partially homologous DNA sequences have *mutS* proteins defective or missing.

8. Process according to claim 7, wherein yeast cells containing the partially homologous DNA sequences have *MLH* genes defective or missing.

9. Process according to claim 1, wherein the eukaryotic cells are of plants.

10. Process according to claim 5, wherein the cells are *pms1* mutants or *msh2* mutants or *pms1 msh2* double mutants.

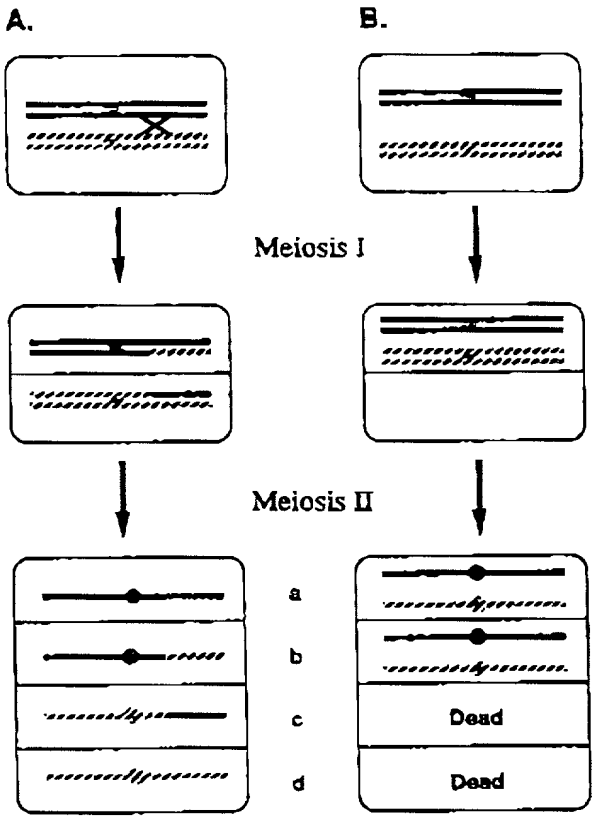

Figure 1

DECLARATION AND POWER OF ATTORNEY FOR U.S. PATENT APPLICATION

( ) Original ( ) Supplemental ( ) Substitute ( ) PCT ( ) Design

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Title: MEIOTIC RECOMBINATION IN VIVO OF PARTIALLY HOMOLOGOUS DNA SEQUENCES

of which is described and claimed in:
( ) the attached specification, or
( ) the specification in the application Serial No. _____ filed _____;
and with amendments through _____ (if applicable), or
(X) the specification in International Application No. PCT/ GB97/00875, filed 27 March 1997, and as amended on 5 June 1998 (if applicable).

I hereby state that I have reviewed and understand the content of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge my duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim priority benefits under Title 35, United States Code, §119 (and §172 if this application is for a Design) of any application(s) for patent or inventor's certificate listed below and have also identified below any application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

COUNTRY	APPLICATION NO.	DATE OF FILING	PRIORITY CLAIMED
US	60/014,490	1 April 1996	X

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not dislcosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application.

APPLICATION SERIAL NO.	U.S. FILING DATE	STATUS: PATENTED, PENDING, ABANDONED

And I hereby appoint John T. Miller, Reg. No. 21,120; Michael R. Davis, Reg. No. 25,134; Matthew M. Jacob, Reg. No. 25,154; Jeffrey Nolton, Reg. No. 25,408; Warren M. Cheek, Jr., Reg. No. 33,367; Nils E. Pedersen, Reg. No. 33,145 and Charles R. Watts, Reg. No. 33,142, who together constitute the firm of WENDEROTH, LIND & PONACK, L.L.P., attorneys to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith.

I hereby authorize the U.S. attorneys named herein to accept and follow instructions from Stevens Hewlett & Perkins as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and myself. In the event of a change in the persons from whom instructions may be taken, the U.S. attorneys named herein will be so notified by me.

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I further declare that all statements made herein of my own knowledge are true, and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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5th Inventor _____ Date _____

6th Inventor _____ Date _____

7th Inventor _____ Date _____

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